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Reconstruction and in silico analysis of the MAPK signaling pathways in the human blood fluke, *Schistosoma japonicum*

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Abstract At present, little is known about signal transduction mechanisms in schistosomes, which cause the disease of schistosomiasis. The mitogen-activated protein kinase (MAPK) signaling pathways, which are evolutionarily conserved from yeast to *Homo sapiens*, play key roles in multiple cellular processes. Here, we reconstructed the hypothetical MAPK signaling pathways in *Schistosoma japonicum* and compared the schistosome pathways with those of model eukaryote species. We identified 60 homologous components in the *S. japoncium* MAPK signaling pathways. Among these, 27 were predicted to be full-length sequences. Phylogenetic analysis of these proteins confirmed the evolutionary conservation of the MAPK signaling pathways. Remarkably, we identified *S. japonicum* homologues of GTP-binding protein β and α-I subunits in the yeast mating pathway, which might be involved in the regulation of different life stages and female sexual maturation processes as well in schistosomes. In addition, several pathway member genes, including ERK, JNK, Sja-DSP, MRAS and RAS, were determined through quantitative PCR analysis to be expressed in a stage-specific manner, with ERK, JNK and their inhibitor Sja-DSP markedly upregulated in adult female schistosomes.

Keywords: *Schistosoma japonicum*; Mitogen-activated protein kinase signaling pathways; Bioinformatics

1. Introduction

Schistosomiasis remains one of the most serious tropical parasitic diseases; at least 200 million people are currently afflicted in 76 countries of the world and a further 500–600 million people are at risk of infection from the three principal species affecting humans, *Schistosoma japonicum*, *S. mansoni* and *S. haematobium* [1,2]. *S. japonicum*, the Asian schistosome, causes intestinal schistosomiasis in China, Japan, Philippines and Indonesia. A nationwide schistosomiasis survey carried out in 2003 indicated that there were still more than 800 000 people infected with *S. japonicum* in China [3].

Mitogen-activated protein kinases (MAPKs) are pivotal transmitters of extracellular signals such as hormones, cytokines, growth factors, and those resulting from various environmental stresses [4]. The MAPK signaling pathways are evolutionarily conserved among eukaryotes and organized as a three-kinase module, consisting of a MAPK, a MAPK kinase (MAP2K/MKK) and a MAPK kinase kinase (MAP3K/MKKK). MAPKs, the last kinases in the cascade are activated by MKKs through dual phosphorylation of conserved threonine and tyrosine residues in a Thr-X-Tyr motif (where X is glutamate, proline or glycine). The MKKs are themselves phosphorylated and activated by serine/threonine kinases that function as MKKKs. Once activated, MAPKs can mediate key cellular processes including cell differentiation, division and death through phosphorylation and regulation of a wide range of substrates including transcription factors, membrane and cytoplasmic proteins as well as other protein kinases [5].

It is likely that the MAPK signaling pathways, which are conserved from yeast to *Homo sapiens*, may be involved in the regulation of the complex life cycle and host–parasite interactions in *S. japonicum*. The MAPK signaling pathways are also implicated in sexual maturation of female schistosomes and egg production, with host inflammatory responses to the eggs representing the cardinal pathological basis of schistosomiasis [6,7]. It is known that, once in copula, the male worm transmits chemical or mechanical signals to regulate the female gene expression in a stage- and tissue-specific manner. Although the molecular mechanism of this process is inadequately understood, Schussler et al. [8] identified three putative signal transducing molecules, RAS, MAPK and a GAP protein in *S. mansoni*, and investigated their potential roles in male–female interactions. They found that these three conserved members of the RAS-ERK signaling pathway are developmentally regulated and are involved in the maturation of the female parasite.

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The mechanisms of cellular signaling transduction in schistosomes remain poorly understood and, moreover, previous studies of signaling processes in schistosomes did not focus in a comprehensive manner on MAPK signaling. Recently, pioneering studies of the transcriptome and proteome of *S. japonicum* and *S. mansoni* have generated very large numbers of expressed sequence tags (EST) [9,10]. There are now about 100,000 *S. japonicum* ESTs in the public domain and these include ~8500 potentially protein-encoding cDNAs, over 3500 predicted full-length cDNAs with potential ORFs, and 3273 proteins identified by mass spectrometry and other proteomics methods. These sequences represent a rich resource which can now be utilized to reconstruct signaling and other pathways to accelerate identification of new intervention targets.

In this report, using bioinformatics approaches we have attempted to reconstruct the MAPK signaling pathways of *S. japonicum* and define key members of the pathways. As well as we have compared and contrasted the schistosome pathways with those of model eukaryote species including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Homo sapiens*. We believe that a better understanding of MAPK signaling pathways regulating proliferation and differentiation processes in schistosomes would certainly accelerate our understanding of parasite pathogenesis and facilitate development of novel treatments.

2. Materials and methods

2.1. Database generation

2.1.1. Schistosome protein database. *S. japonicum* ESTs from six different life stages, assembly cluster data and database of proteins predicted to be encoded by the EST cluster data were downloaded from http://schistosoma.chgc.sh.cn/sj-proteome/download/download.php, maintained by the China National Humane Genome Center (CHGC) at Shanghai. In addition, we collected another 368 *S. japonicum* proteins from NCBI (until 23 July 2005). We combined the two groups of *S. japonicum* protein data as a new protein database with about 8820 protein sequences. The schistosome protein database is freely accessible online at http://www.scbit.org/eng/mapk.php.

2.1.2. MAPK protein database. *H. sapiens*, *Rattus norvegicus*, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, *Danio rerio* and *Plasmodium falciparum* protein sequences with known involvement in the MAPK signaling pathways were retrieved from the KEGG pathway database [11]. Moreover, sequences involved in the pathways of all organisms were collected from Entrez protein by text-based searching, taking advantage of diverse combination of words related to the pathway members. The sequences were somewhat redundant due to the large scale collection from NCBI Entrez protein. Sequences with lengths less than 100 amino acids were removed by using perl scripts. For this report, we refer to these proteins and sources as the MAPK Protein Database; this database is freely accessible online at http://www.scbit.org/eng/mapk.php.

2.2. Retrieval of MAPK signaling pathways sequences from the schistosome protein database

In order to identify homologues involved in the MAPK signaling pathways in *S. japonicum*, we used the MAPK Protein Database mentioned above as the query and carried out a stand-alone BlastP program [12] search against the schistosome protein database with an *E*-value cutoff of e^{-5}. Further, to confirm the authenticity of the putatively orthologous proteins and to eliminate paralogues, we undertook a reverse BlastP similarity search using the schistosome protein database against the NR (non-redundant) database of NCBI with the same cutoff of *E*-value. In this way, the bi-directional best hit (BBH) results from the schistosome protein database were putatively assigned as candidate members of *S. japonicum* MAPK signaling pathways. Subsequently, by a thorough text-based mining review and examination of domains and motifs of these pathway candidates using InterPro database [13] and stand-alone InterProScan [14], we have identified the prospective *S. japonicum* orthologues in the MAPK signaling pathways.

2.3. Molecular mass and physico-chemical properties

Molecular mass and theoretical *pI* (isoelectric point) for the potential full-length *S. japonicum* proteins were predicted using the ProtParam tool of ExPaSy (http://au.expasy.org/tools/protparam.html).

2.4. Molecular phylogenies

Multiple sequence alignments were assembled with the aid of the Clustal W1.83 program [15]; the results were handled by Gblocks_0.91b program [16] to eliminate large gaps, and refined manually. We used two programs for phylogenetic analyses. The Phylip3.6a3 package [17] was used to generate neighbor-joining trees with the Kimuru model of amino acid substitution, and the Mega (version 3.1) suite of programs [18] was accessed for analysis of some multiple gene families. The resulting trees were bootstrapped (1000 bootstrap replicates) to obtain information on statistical significance. The resulting trees were visualized and displayed using TreeView (version 1.6.6) [19].

2.5. Real time quantitative RT-PCR assay

Total RNA of *S. japonicum* from four different life stages, including cercariae, schistosomula, female and male adults, and eggs was extracted with TRIzol reagent. The concentrations and purity of the RNA in each sample were determined by spectrometry at 260 and 280 nm. The RNA samples were reverse transcribed to cDNA using oligo (dT) (Qiagen, California) and Superscript II reverse transcriptase (Invitrogen). Primers for selected genes (primers were designed with Primer Premier5 and synthesized by TaKaRa Biotechnology (Dalian) Co., Ltd.) are presented in Supplementary Table 1. Real time PCR reactions, using 2 × AB gene SYBR Rox Mix (Applied Biosystems), were accomplished in a 7900HT real-time thermal cycler (Applied Biosystems) driven by GeneAmp 7900HT sequence detection system software (Perkin–Elmer Corp., Foster City, CA). According to the manufacturer's instructions, the program was as follows: one cycle of 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. *S. japonicum* tubulin (GenBank Accession No.: AY815746), a constitutively expressed structure protein was used as an internal control gene to normalize the expression levels between samples [20]. All standards and samples were tested in triplicate. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta C_t}$ method [21]. The threshold cycle (C_t) was defined as the cycle at which the fluorescence signal was statistically significant above background level and was inversely proportional to the initial template copy number. Therefore, $\Delta C_t = C_t$ of the target gene $- C_t$ of the internal control gene.

3. Results and discussion

3.1. Establishing the S. japonicum MAPK sequence database

A protein database of sequences considered to be members of the MAPK signaling pathways (23 July 2005) was established. We collected more than 12 000 sequences from the Entrez Protein and KEGG signaling pathway database and splice variants as well as partial sequences are also included in our analysis. Our bioinformatics assisted interrogation of this database identified 224 candidates as prospective components of MAPK signaling pathways in *S. japonicum*.

3.2. Generalized features of the S. japonicum MAPK signaling pathways

In this report, we have investigated the MAPK signaling pathways in *S. japonicum* using a bioinformatics approach and determined 60 potential components, representing a large majority of the established MAPK signaling pathway members

Table 1
Schistosoma japonicum members of the MAPK signaling pathways based on homology searches against the *Homo sapiens*, *Rattus norvegicus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Danio rerio* MAPK signaling pathways

	Group	*S. japonicum* Gene	*S. japonicum* Accession No.	*S. japonicum* no.	*Homo sapiens*		*Rattus norvegicus*		*Drosophila melanogaster*		*Caenorhabditis elegans*		*Saccharomyces cerevisiae*		*Danio rerio*	
					E value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)
1	MAP4K	MAP4K 2/3	AAX28389	SJCHGC05877	1.00E − 88	56.99	8.00E − 88	56.11	4.00E − 88	46.70	7.00E − 63	45.11	NA		2.00E − 93	52.35
2		STK3/4 (serine/threonine kinase 3/4)	AAX27921	SJCHGC08035	5.00E − 68	67.03	5.00E − 68	67.03	2.00E − 66	65.95	3.00E − 66	66.67	NA		1.00E − 61	45.08
3		PAK (p21-activated kinase)	AAX25818	SJCHGC04783	1.00E − 82	66.52	4.00E − 84	66.52	8.00E − 86	69.23	1.00E − 58	50.22	3.00E − 57	46.30	NA	
4	MAP3K	MAP3K	AAW27786	SJCHGC07240	3.00E − 27	38.99	7.00E − 29	40.25	4.49E − 01	44.91	NA		NA		1.00E − 21	37.89
5	MAP2K	MKK4	AAX27485	SJCHGC03483	9.00E − 39	59.70	NA		1.00E − 34	63.30	6.00E − 25	46.34	NA		7.00E − 38	59.70
6		MKK7	AAX30509	SJCHGC04258	2.00E − 28	42.11	4.00E − 28	42.77	NA		5.00E − 23	44.12	NA		1.00E − 25	38.41
7		MKK3/6	AAP06412	SJCHGC05376	4.00E − 37	47.19	8.90E − 38	48.31	2.00E − 29	42.70	1.00E − 29	44.71	NA		2.00E − 34	46.11
8			AAX25822	SJCHGC04085	5.00E − 24	37.10	6.00E − 24	36.56	6.00E − 24	35.18	3.00E − 17	29.44	NA		8.00E − 24	38.54
9	MAPK	ERKs/Fus3 (yeast)	AAT02418	SJCHGC02286	1.00E − 141	67.93	2.00E − 142	68.13	1.00E − 139	67.15	1.00E − 137	66.87	6.00E − 89	47.85	2.00E − 138	67.15
10		JNKs	AAW27550	SJCHGC05891	1.00E − 146	70.91	4.00E − 147	71.63	1.00E − 149	70.72	1.00E − 137	63.97	NA		NA	
11		NLK (nemo like kinase)	ABA40346	SJCHGC09000	1.00E − 78	50.99	1.00E − 76	43.00	1.00E − 75	44.00	1.00E − 79	45.00	NA		1.00E − 76	46.00
12			AAW24716	SJCHGC09514	1.00E − 67	57.26	1.00E − 67	57.26	4.00E − 68	57.76	4.00E − 65	57.57	NA		4.00E − 68	55.95
13	RAS	RAS	AAW24814	SJCHGC09408	8.00E − 69	78.71	5.90E − 69	78.71	8.00E − 64	79.73	2.00E − 63	77.27	2.00E − 47	57.58	1.10E − 64	76.13
14	interacting	MRAS	AAW25576	SJCHGC06290	5.00E − 59	58.89	2.90E − 59	61.54	NA		6.00E − 61	57.87	NA		NA	
15	proteins	RAP1	AAX27721	SJCHGC06295	2.00E − 70	81.70	1.00E − 70	81.70	NA		NA		NA		NA	
16			AAW25200	SJCHGC03962	1.00E − 60	64.67	1.00E − 59	64.13	NA		NA		NA		NA	
17		GRB2 (adaptor protein)	AAP06014	SJCHGC05896	1.00E − 56	57.82	8.60E − 53	45.81	2.00E − 51	52.43	2.00E − 48	47.12	NA		2.00E − 41	37.45
18		SOS	AAX30854	SJCHGC08194	4.00E − 08	33.07	5.00E − 07	31.50	5.00E − 10	30.71	7.00E − 07	27.86	NA		1.00E − 08	31.78
19		GTPase activating protein (GAP)	AAX26455	SJCHGC08700	6.00E − 18	30.63	4.00E − 18	30.63	1.00E − 16	33.33	1.00E − 15	31.05	NA		1.00E − 15	31.05
20		NF1(neurofibromatosis 1)	AAX26220	SJCHGC09051	1.00E − 178	63.90	5.00E − 178	63.17	NA		NA		NA		NA	
21	CDC42/RAC	CDC 42	AAW27693	SJCHGC06334	7.00E − 84	77.89	6.50E − 84	78.53	6.00E − 83	77.49	NA		1.00E − 78	71.73	NA	
22	interacting proteins	RAC 2	AAW24792	SJCHGC01385	3.00E − 78	71.88	7.00E − 78	71.35	1.00E − 79	73.44	1.00E − 68	69.44	NA		NA	
23	MAPK	DSP	NA	Sja-DSP	1.00E − 34	29.75	3.00E − 16	38.06	3.00E − 13	35.97	NA		NA		2.00E − 18	38.16
24	regulating	MAPK phosphatase	AAW25026	SJCHGC01353	8.00E − 25	41.25	1.00E − 24	41.25	6.00E − 21	37.68	NA		NA		2.00E − 23	38.51
25	proteins	Protein tyrosine	AAX28613	SJCHGC04925	2.00E − 40	39.37	3.70E − 40	34.55	NA		NA		NA		NA	
26		phosphatase (PTP)	AAX30135	SJCHGC00531	5.00E − 40	49.04	6.00E − 40	48.41	3.00E − 43	48.78	NA		NA		3.00E − 39	49.04
27		PTP (non-receptor type)	AAX28494	SJCHGC07177	NA		NA		2.00E − 45	62.50	NA		NA		NA	
28		Protein phosphatase 2C	AAW27443	SJCHGC09402	9.00E − 91	52.98	4.80E − 91	52.65	NA		NA		NA		NA	
29			AAX28472	SJCHGC03888	1.00E − 54	56.42	1.00E − 54	55.87	NA		NA		NA		NA	
30		Protein phosphatase 2B, regulatory subunit (calcineurin B)	AAO59418	SJCHGC05872	6.00E − 81	85.88	3.00E − 81	85.88	NA		NA		NA		NA	
31		PP2B, catalytic subunit (calcineurin A)	AAX26394	SJCHGC08224	7.00E − 45	74.56	7.00E − 45	74.56	NA		NA		NA		NA	
32		Protein phosphatase 5	AAW27026	SJCHGC03179	2.00E − 65	52.34	3.70E − 62	60.12	NA		NA		NA		NA	
33		AKT1	AAX25998	SJCHGC09382	2.00E − 83	62.66	2.50E − 84	63.09	NA		NA		NA		NA	

(*continued on next page*)

Table 1 (*continued*)

	Group	*S. japonicum* Gene	*S. japonicum* Accession No.	*S. japonicum* no.	*Homo sapiens*		*Rattus norvegicus*		*Drosophila melanogaster*		*Caenorhabditis elegans*		*Saccharomyces cerevisiae*		*Danio rerio*	
					E value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)	*E* value	Identity (%)
34		Heat shock 70 kDa	AAC00519	SJCHGC06292	0.00	59.67	0.00	63.67	NA		NA		NA		NA	
35		protein	AAW24917	SJCHGC06312	0.00	74.31	NA		NA		NA		NA		NA	
36			AAX26465	SJCHGC05757	6.00E − 87	76.92	8.00E − 87	76.92	NA		NA		NA		NA	
37	MAPKAPK	Ribosomal protein S6	AAX27891	SJCHGC05738	1.00E − 88	57.87	8.00E − 83	52.77	4.00E − 71	50.69	NA		NA		NA	
38		kinase	AAX26731	SJCHGC08230	2.00E − 56	68.35	2.00E − 56	68.35	8.00E − 52	62.50	NA		NA		NA	
39		MAPKAPK2	AAX25782	SJCHGC06952	3.00E − 21	37.41	2.00E − 21	38.10	NA		4.00E − 21	37.32	NA		2.00E − 22	37.25
40	Scaffolding /	Filamin A, alpha	AAX27515	SJCHGC07767	2.00E − 26	51.64	NA		NA		NA		NA		NA	
41	Anchoring	JIP3 (JNK- interacting protein 3)	AAX28611	SJCHGC07771	3.00E − 32	42.00	1.00E − 32	43.00	NA		1.00E − 28	37.00	NA		NA	
42		arrestin, beta	AAX26296	SJCHGC09071	2.00E − 42	51.83	9.00E − 51	45.96	NA		NA		NA		2.30E − 51	44.85
43			AAX25687	SJCHGC08572	8.00E − 30	65.91	1.00E − 29	65.91	NA		NA		NA		1.00E − 29	64.77
44		14-3-3 protein	AAW27287	SJCHGC01759	9.00E − 85	66.67	2.00E − 82	64.00	7.00E − 85	67.00	9.00E − 85	67.00	NA		1.00E − 84	66.00
45			AAD56715	SJCHGC01843	1.00E − 75	63.11	2.00E − 75	63.11	1.00E − 78	67.08	1.00E − 78	66.39	NA		2.00E − 77	65.02
46		14-3-3 epsilon	AAW26747	SJCHGC01755	2.00E − 74	57.81	7.00E − 74	57.81	8.00E − 76	59.75	4.00E − 73	58.02	NA		4.00E − 75	58.82
47			AAW25339	SJCHGC06291	3.00E − 71	61.09	4.00E − 69	60.25	1.00E − 74	60.56	2.00E − 66	58.62	NA		3.00E − 72	61.51
48	MAPK Substrates	Phospholipase A2	AAX25779	SJCHGC09449	1.00E − 30	54.84	NA		NA		NA		NA		NA	
49	Receptors	EGFR	AAX27836	SJCHGC05493	1.00E − 74	53.38	9.80E − 75	44.51	6.00E − 77	53.24	2.00E − 49	40.07	NA		NA	
50		FGFR	AAX24939	SJCHGC03615	2.00E − 27	41.76	2.00E − 27	41.62	8.00E − 24	35.64	NA				3.00E − 27	42.86
51	Others	Caspase	AAW26244	SJCHGC00800	8.00E − 50	42.49	8.00E − 50	42.49	NA		NA		NA		NA	
52			AAX25947	SJCHGC04214	3.00E − 43	42.65	6.00E − 42	38.96	NA		NA		NA		NA	
53			AAX25741	SJCHGC03058	7.00E − 40	44.32	1.00E − 37	42.61	NA		NA		NA		NA	
54			AAX26691	SJCHGC07329	1.00E − 26	32.30	1.00E − 25	32.94	NA		NA		NA		NA	
55		G protein alpha	AAW26775	SJCHGC00931	1.20E − 70	42.82	1.20E − 70	42.82	NA		NA		NA		NA	
56		G protein beta	AAW26990	SJCHGC05537	NA		NA		NA		NA		5.00E − 67	37.08	NA	
57		PKA, catalytic subunit	AAW25592	SJCHGC06050	1.00E − 169	81.85	1.00E − 140	83.46	5.00E − 141	85.67	6.00E − 51	39.00	NA		5.00E − 139	82.94
58			AAX27942	SJCHGC06215	2.00E − 104	85.02	5.00E − 105	85.46	1.00E − 106	88.84	8.00E − 106	87.67	NA		7.00E − 105	85.02
59			AAW26015	SJCHGC04411	1.00E − 90	65.02	3.00E − 91	65.78	7.00E − 89	65.60	9.00E − 89	64.54	NA		3.00E − 92	66.67
60		PKC	AAW26316	SJCHGC06499	1.00E − 81	66.35	1.00E − 81	66.35	3.00E − 81	67.77	8.00E − 75	66.36	NA		2.00E − 78	65.40

Note that "NA" means that no data are available in the public domain for the MAPK signaling pathway in that specific organism.

known from other organisms (Table 1). Most were previously unknown in schistosomes, and 27 of them were predicted to be full-length sequences (Supplementary Table 2). On the other hand, we did not (yet) identify several pathway homologues in *S. japonicum*. This ostensible absence may relate to the insufficient sequence information for these orthologues in the schistosome protein database or may reflect the actual absence of these genes from the schistosome genome. However, since there is underway at the CHGC at present a concerted effort to sequence the entire genome of *S. japonicum*, we also undertook a preliminary genome scan of the currently available (but yet unpublished) *S. japonicum* genome sequences for these apparently absent genes, and indeed identified several genes that may be members of the *S. japonicum* MAPK signaling pathways (Fig. 5; and Wang et al., unpublished). It is anticipated that with the release of the complete genome sequences of both *S. japonicum* and *S. mansoni* in the near future, more comprehensive information about signaling pathways in schistosomes will be available, and will allow an even more thorough investigation of their physiology and cell biology.

3.3. *Orthologue analysis of* S. japonicum *signaling pathways components*

In order to decipher more specific and detailed information about the pathway members, we undertook a series of orthologue analyses. These included multiple sequence alignments to identify putative conserved motifs of schistosome orthologues and neighbor-joining trees to place novel schistosome genes accurately within the appropriate family. Taking MAPKs for example, in higher eukaryotes, including humans, five MAPK subgroups have been identified; these are known as ERK1/2, JNK, p38, ERK3/4 and ERK5 [22]. In addition, the nemo-like kinase, NLK, a mammalian relative of *Drosophila* nemo, has been described: NLK negatively regulates the Wnt signaling pathway [23]. We identified four novel potential *S. japonicum* MAPKs, one ERK, one JNK and two NLKs. Three of them were predicted to be full-length sequences. A neighbor-joining tree was generated using a kinase catalytic domain (Fig. 1). The three full-length *S. japonicum* proteins were predicted to represent three of the subgroups. AAW27550 (SJCHGC05891) was orthologous to JNK (including human JNK, *D. melanogaster* and *C. elegans* counterparts: Bsk and JNK1) (bootstrap value of 100%). The others, AAT02418 (SJCHGC02286) and ABA40346 (SJCHGC09000) were orthologous to NLK (98%) and ERK1/2 (100%), respectively. Multiple alignment of the amino acid sequence of *S. japonicum* ERK showed that all the 11 conserved subdomains of serine/threonine kinase [24] were well conserved, including the consensus phosphate anchor ribbon GXGXXGXV (Gly^{24}-Val^{31}) for ATP binding in subdomain I and the invariant Lys^{46} in subdomian II (Fig. 2). The conserved TXY activation site was also found both in *S. japonicum* ERK (TEY) and JNK (TPY) within the phosphorylation lip between kinase subdomains VII and VIII. Moreover, key catalytic and signature residues for MAPKs were also present in the *S. japonicum* orthologues. The signature sequence of MAPKs, (F-X_{10}-R-E-X_{77}-R-D-X-K-X_{14}-C), which is absent from all other classes of protein kinases [25], was also present in this sequence and maps to amino acid positions 51–159. A CD domain, a conserved domain associated with MAPKs docking to their activators (like MAP2Ks), inhibitors (like MAPK phosphatases) and substrates (like

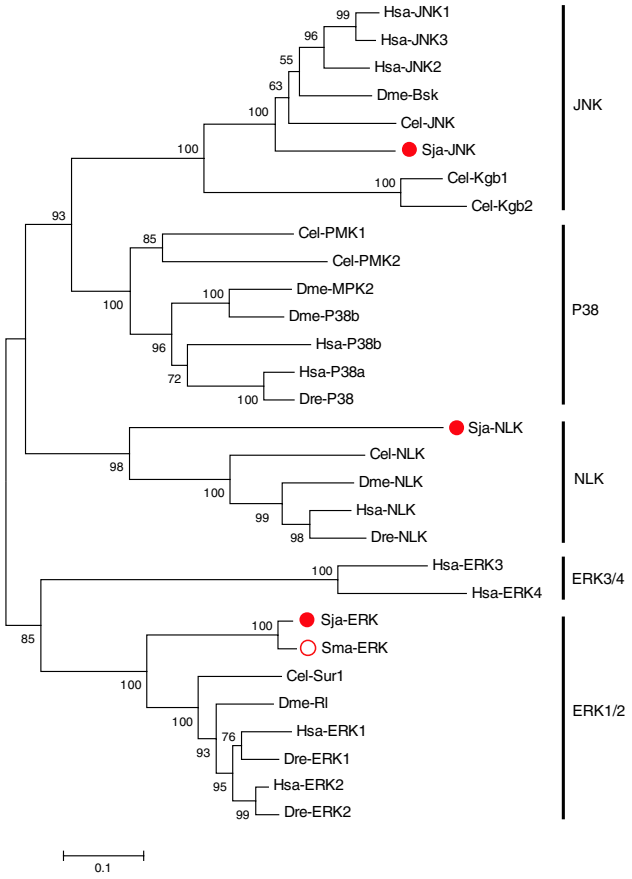

Fig. 1. Phylogenetic analysis of MAPKs generated by the neighbor-joining method based on the alignment of protein domains between *S. japonicum* and other eukaryotes. *S. japonicum* proteins are shown by solid red dots, while open red dots indicate proteins from *S. mansoni*. The bootstrap values are indicated near the corresponding node. The scale bar presents 0.1 amino acid substitutions per position of evolutionary distance. Hsa, *Homo sapiens*; Sja, *Schistosoma japonicum*; Sma, *Schistosoma mansoni*; Dme, *Drosophila melanogaster*; Cel, *Caenorhabditis elegans*; Dre, *Danio rerio*.

MAPKAPK) was located near the C-terminal of the proteins [26]. The CD domain was well conserved in *S. japonicum* ERK despite the substitution of some alkaline amino acids (His for Gln^{308} and Lys for Glu^{319}). These differences may facilitate development of novel inhibitors or agonists of *S. japonicum* ERK.

Our findings also indicate that the MAPK signaling pathways might be central to sexual maturation of the female schistosome. We identified *S. japonicum* homologues of guanine nucleotide binding protein (G protein) β subunit (AAW26990, SJCHGC05537) and α subunit (AAW26775, SJCHGC00931), both predicted to be full-length sequences. Heterotrimeric G proteins, consisting of α, β and γ subunits, act as signal transducers, coupling cellular surface receptors to cytoplasmic effector proteins. G proteins play important roles during sexual development of fungi, including ascomycetes and basidiomycetes [27]. In *S. cerevisiae*, G proteins constitute part of the pheromone signaling mating pathway and the β/γheterodimer, which is activated by dissociation from the α subunit, can transmit signal to a MAPK cascade that finally phosphorylates downstream targets responsible for regulation of the haploid mating process [28]. Also, the α subunit may

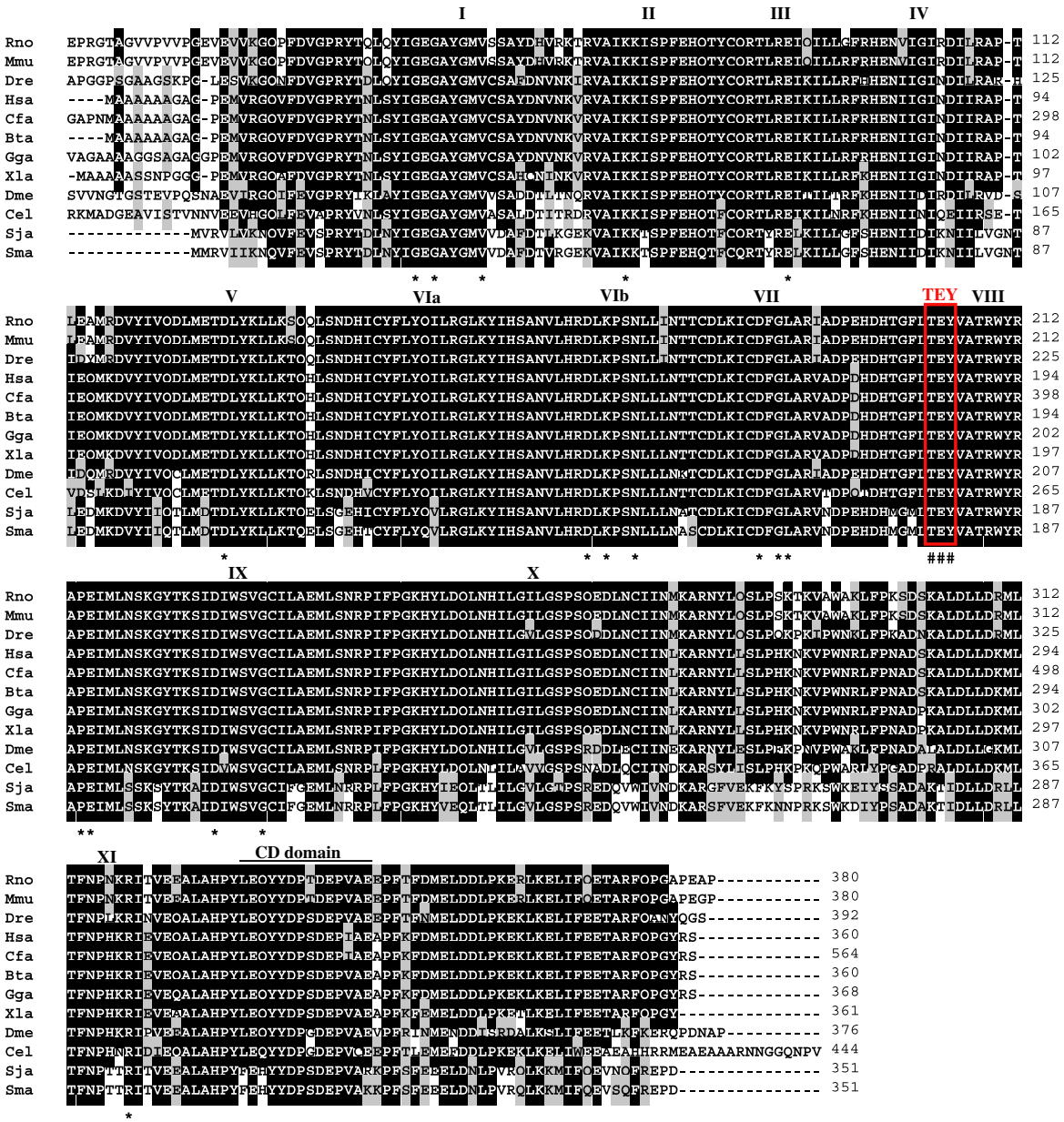

Fig. 2. Alignment of predicted amino acid sequences of the *S. japonicum* ERK with other ERKs. Identical (black) or chemically similar (gray) residues to *S. japonicum* ERK are indicated by shading. The 11 subdomains of the serine–threonine protein kinase catalytic domain are indicated with Roman numerals. Hallmark conserved catalytic residues are labeled with asterisks (below). The red column masked with "#" represents the conserved dual phosphorylation sites TEY (Thr^{178}-Tyr^{180}). The position of the CD domain is indicated with an over head line. Sja, *Schistosoma japonicum*; Sma, *Schistosoma mansoni*; Hsa,*Homo sapiens* (Accession No.: NP_002736.3); Dme, *Drosophila melanogaster* (Accession No.: AAL48618.1); Cel,*Caenorhabditis elegans* (Accession No.: NP_497847.2); Xla, *Xenopus laevis* (Accession No.: P26696); Rno, *Rattus norvegicus* (Accession No.: CAA46318); Mmu, *Mus musculus* (Accession No.: NP_036082.1); Cfa, *Canis familiaris* (Accession No.: XP_534770.1); Gga,*Gallus gallus* (Accession No.: NP_989481.1); Dre, *Danio rerio* (Accession No.: AAH97073.1); Bta, *Bos taurus* (Accession No.: CAA78467.1).

bind to MAPK and negatively regulate the mating signal [29,30]. As for parasites, G proteins are also involved in switching to sexual development (gametocytogenesis) of *Plasmodium falciparum* [31] and regulation of the choice between free-living and parasitic life cycles of *Strongyloides stercoralis* [32]. However, little is known about signaling function or transduction mechanisms of G proteins in schistosomes. Only an α subunit of G protein has been characterized from *S. mansoni* and the gene was developmentally controlled [33]. Herein, the *S. japonicum* G protein α subunit shared 49% and 48% identities to *S. stercoralis* gpa2 and gpa3, respectively, and contained all the conserved motifs responsible for guanine nucleotide binding and hydrolysis as well as an N-terminal myristoylation site (Supplementary Fig. 7). The identification of two subunits of the G protein and a number of MAPK signaling pathway members could indicate that the MAPK signaling pathways in *S. japonicum* participate in the regulation of different life stages and female sexual differentiation processes as well. Similarly, we identified other members of the pathway including MAP2Ks, MAP3Ks, MAP4Ks, RAS small

G protein family members, MAPK phosphatases, scaffolding or anchoring proteins in the *S. japonicum* transcriptome data and detailed information about them can be found in the Supplementary material.

At present, only one drug, praziquantel is available for the treatment of all forms of schistosomiasis [34]. Because of its widespread use and reports of the development of drug resistance [35,36], there is a pressing need to identify new drug targets. We anticipate that blockade of the MAPK signaling pathways would represent a powerful new approach to anti-schistosomal chemotherapy in like manner to the substantial focus on MAPK inhibitors as medications for the treatment of inflammatory diseases [37,38]. In this report, we performed a search of the 60 *S. japonicum* MAPK signaling pathways members against the therapeutic target database (TTD) [39] and listed all possible drug targets homologues in this signaling cascade (Supplementary Table 3). The Therapeutic Target Database has collected about 1174 known therapeutic protein and nucleic acid targets, 1251 drugs or ligands directed at these targets (as of September 2004), as well as information about the targeted disease and signaling pathway. The search revealed 19 therapeutic target homologues in the *S. japonicum* MAPK signaling pathways.

3.4. *Phylogenetic analysis of* S. japonicum *MAPK signaling pathway members*

We performed phylogenetic analyses using several conserved full-length *S. japonicum* proteins in the MAPK signaling pathways. The tree for CDC42 confirmed that *S. japonicum* (AAW27693, SJCHGC06334) and *S. mansoni* (Accession No.: AAN77582.1) share close identity to orthologues in *C. elegans* and the filarial nematode *Brugia malayi* (Fig. 3A). The tree for one 14-3-3 protein, AAW27287 (SJCHGC01759), which is recognized as a potential anti-schistosomal vaccine candidate [40,41], indicated that *Schistosoma* 14-3-3, including orthologues from *S. japonicum*, *S. mansoni* (Accession No.: Q26540) and *S. bovis* (Accession No.: AAT39382.1) shared closest identity to an orthologue in the cestode genus, *Echinococcus* (Fig. 3B). The same trend was apparent with ERK, JNK, HSP70 and MRAS (not shown). *Schistosoma*, as a representative genus of the Phylum Platyhelminthes, diverged early from other bilateral metazoans [42]. Consequently, the sequence information described here for schistosome orthologues in these signaling pathways is likely to be of value also in investigations of early metazoan evolution.

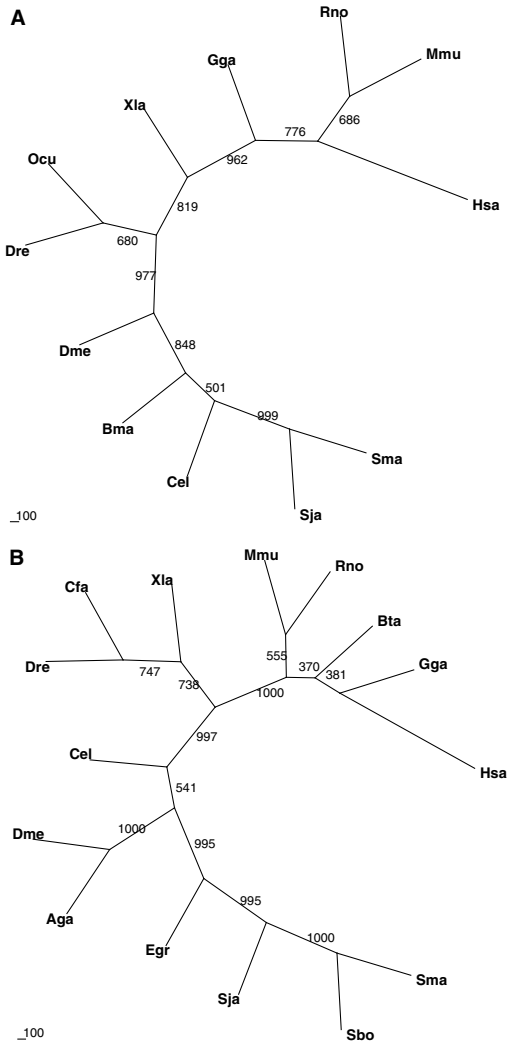

Fig. 3. Phylogenetic analysis with neighbor-joining trees using Phylip 3.6a: (A) CDC42, and (B) 14-3-3 proteins. Numbers on the branches represent bootstrap support values (1000 bootstrap replicates). Hsa, *Homo sapiens*; Rno, *Rattus norvegicus*; Dre, *Danio rerio*; Dme, *Drosophila melanogaster*; Cel, *Caenorhabditis elegans*; Sja, *Schistosoma japonicum*; Sma, *Schistosoma mansoni*; Sbo, *Schistosoma bovis*; Xla, *Xenopus laevis*; Gga,*Gallus gallus*; Ocu, *Oryctolagus cuniculus*; Bma, *Brugia malayi* (filarial worm); Egr, *Echinococcus granulosus* (cestode); Aga, Anopheles gambiae; Cfa, *Canis familiaris*; Bta, *Bos taurus*.

3.5. *Stage specific expression of* S. japonicum *MAPK signaling pathway members*

We used real time PCR to quantify the expression levels of MAPK signaling mRNAs in several developmental stages of the schistosome life cycle. Fig. 4 presents the relative expression levels of ERK, JNK, Sja-DSP, MRAS and RAS. As expected, all five of these MAPK pathway components were expressed in most, if not all, developmental stages of the schistosome examined here. Furthermore, we observed that the transcription levels of ERK, JNK and their inhibitor Sja-DSP, were all markedly upregulated in adult female schistosomes, as compared to male worms. This suggests that the classic ERK and JNK pathways of the schistosome might be involved in female sexual maturation processes.

We identified two RAS proteins. MRAS was expressed at higher levels in female worms and eggs than male worms (approximately threefold and 6.4-fold, respectively), which was in accordance with the quantitative RT-PCR results of SmRAS reported by Osman et al. [43]. However, the other *S. japonicum* RAS protein (AAW24814, SJCHGC09408) exhibited a more or less steady level throughout the developmental stages. This suggests that, as was the case of ERK and JNK, the *S. japonicum* MRAS protein could be involved in vitelline cell maturation and egg production of female worms while the other *S. japonicum* RAS protein might play a more general role in regulating normal growth and development of the parasite.

3.6. *Reconstruction of* S. japonicum *MAPK signaling pathways*

Taking advantage of known MAPK signaling pathways from different model organisms, including human, rat, *Drosophila* and *C. elegans*, we reconstructed potential MAPK

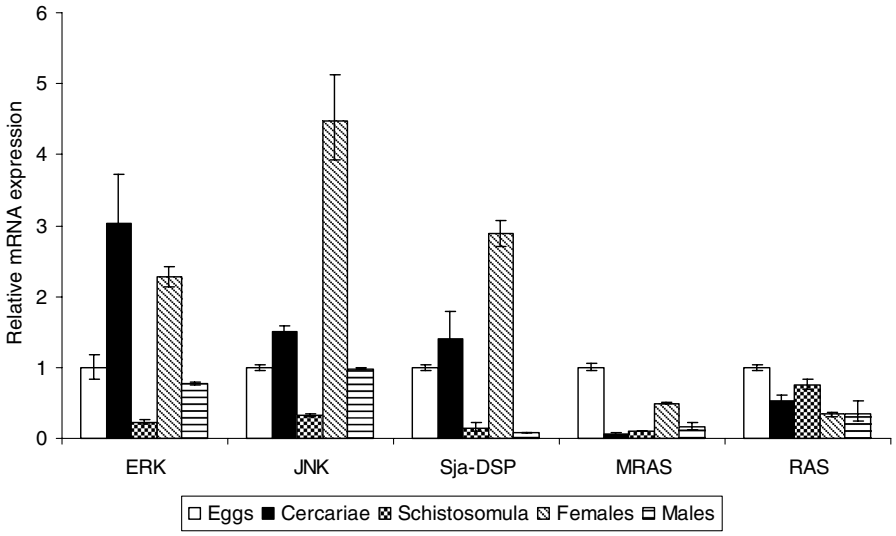

Fig. 4. Real time RT-PCR analysis of MAPK signaling mRNAs. Data (means ± S.E.M.) were calculated by the $2^{-\Delta\Delta C_t}$ method. *S. japonicum* tubulin mRNA was used as reference to normalize the expression level and the mean value obtained from the egg stage was set at one.

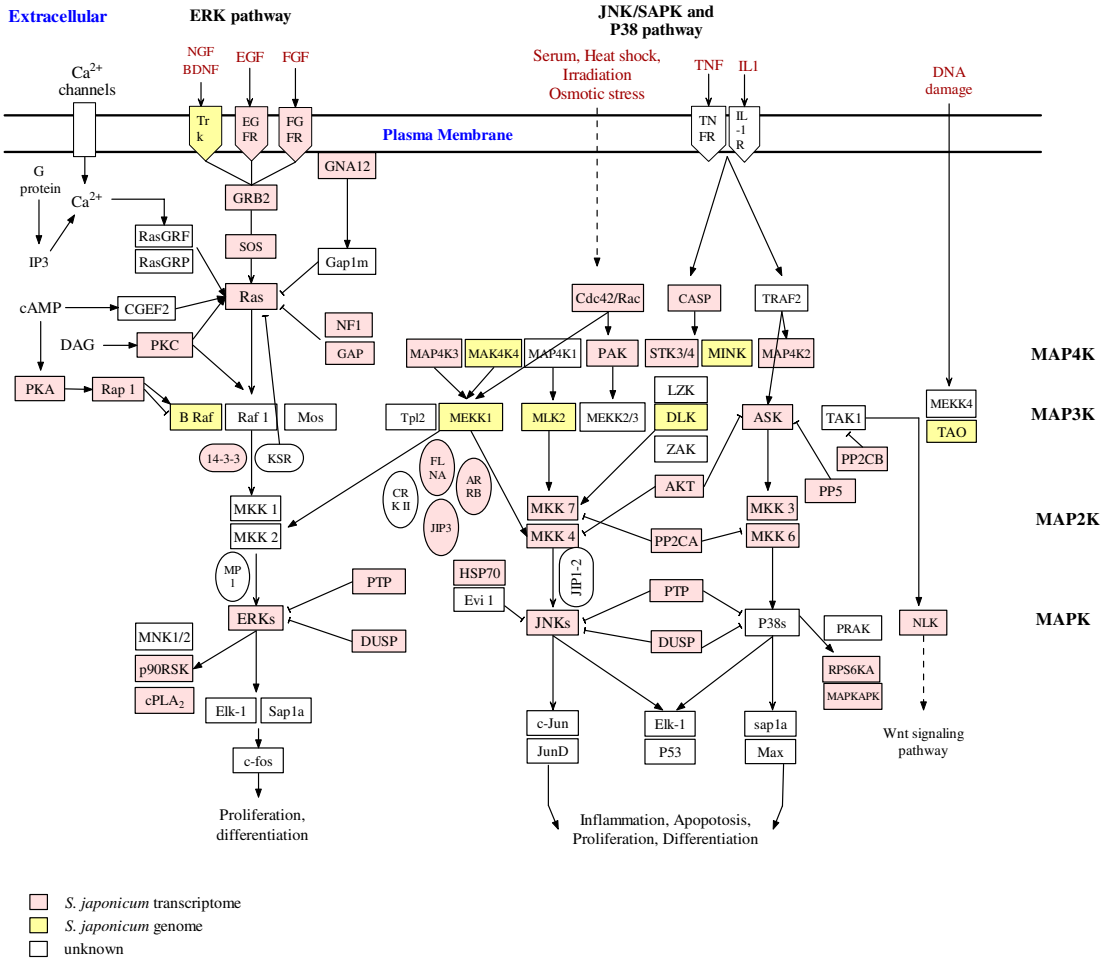

Fig. 5. Reconstruction of the hypothetical mitogen-activated protein kinase pathways in *Schistosoma japonicum* based on sequence identity to known MAPK signaling pathways. Proteins shown in red represent orthologues found in *S. japonicum* transcriptome data, whereas proteins in yellow indicate those found through *S. japonicum* genome scanning. The proteins indicated in white have not (yet) been identified in the *S. japonicum* MAPK signaling pathways. This map is based on the KEGG pathway (No: 04010) for the human MAPK pathway in KEGG.

signaling pathways in *S. japonicum* (Fig. 5). These hypothetical pathways suggest that most members of the pathways in these model organisms are present in *S. japonicum* and thereby indicate that the signaling pathways are highly conserved among higher and lower eukaryotes including the Platyhelminthes. The MAPK signaling pathways are some of the best-characterized signaling systems. In mammals, at least five MAPK cascades have been described; these include the extracellular signal-regulated kinase (ERK) cascade, which regulates cell growth and differentiation, the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and the p38 MAPK cascades, which function mainly in stress responses like inflammation and apoptosis [44]. In *D. melanogaster* and *C. elegans*, the pathways are also responsible for critical cellular and developmental processes [45–47].

In *S. japonicum*, the MAPK signaling pathways might play an essential role in responding both to host and self signals. As for host-derived signals, the identification of growth factor receptors, which share high similarity with human counterparts, implies that the parasite can accept host signals for cell proliferation and development through the ERK cascade. In response to host extracellular stimuli such as growth factors or mitogens, the *S. japonicum* ERK cascade can be predicted (based on the orthologues we have identified here) to consecutively recruit GRB2, SOS, and then, in turn, activate RAS, Raf, MKK1/2 and ERKs. The *S. japonicum* JNK/SAPK signaling pathway may likely respond to host stress such as inflammatory cytokines and tumor necrosis factor (TNF) receptor signaling, or environmental changes like heat shock, osmotic stress and UV irradiation through cascade phosphorylation of CDC42/RAC, MAP4Ks, MAP3Ks, MKK4/7 and JNKs. It is known that host immune factors may likewise influence schistosome development. Amiri et al. [48] observed that the host-derived proinflammatory cytokine TNF alpha acted as a positive signaling regulator of fecundity for female schistosomes and of schistosome reproduction and egg excretion. Although we have not detected a p38 MAPK like sequence in *S. japonicum*, the presence of MKK3/6 and MKK4, the upstream MAP2Ks responsible for the activation of p38 MAPKs (in humans and other species), suggests that the p38 MAPK signaling pathways might also be present in *S. japonicum* and play a similar role to the JNK/SAPK signaling pathway. Moreover, the identification of *S. japonicum* G protein subunit homologues further suggests that the MAPK signaling pathways in *S. japonicum* participate in sexual differentiation processes of female worms through responding to self signals triggered by male worms.

4. Conclusions

MAPK signaling pathways have central roles in the adaptive response of eukaryotic cells to a wide range of stimuli. This study has provided insights into the MAPK signaling pathways of *S. japonicum*. We have investigated the MAPK signaling pathway system in *S. japonicum* using a bioinformatics protocol and identified or predicted most of the pathway components in the parasite. We have used multiple alignment and phylogenetic analysis to assign novel schistosome genes within the appropriate family of higher eukaryote orthologues. One shortcoming of establishing signaling pathways based on gene sequence mining is that the approach cannot discover novel pathway elements and their associated topology. Nevertheless, the sequence information identified here can now be more thoroughly investigated, especially in relation to how schistosomes respond to stresses triggered by the host, and how male signals activate female maturation and egg production. More importantly, the in silico techniques employed here enabled rapid identification of probable signaling pathways, and can pave the way for high throughput screening of potential targets for anti-schistosome interventions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.05.055.

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